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Evidence against a germ plasm in the milkweed bug *Oncopeltus fasciatus*, a hemimetabolous insect

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Summary

Primordial germ cell (PGC) formation in holometabolous insects like *Drosophila melanogaster* relies on maternally synthesised germ cell determinants that are asymmetrically localised to the oocyte posterior cortex. Embryonic nuclei that inherit this “germ plasm” acquire PGC fate. In contrast, historical studies of basally branching insects (Hemimetabola) suggest that a maternal requirement for germ line genes in PGC specification may be a derived character confined principally to Holometabola. However, there have been remarkably few investigations of germ line gene expression and function in hemimetabolous insects. Here we characterise PGC formation in the milkweed bug *Oncopeltus fasciatus*, a member of the sister group to Holometabola, thus providing an important evolutionary comparison to members of this clade. We examine the transcript distribution of orthologues of 19 *Drosophila* germ cell and/or germ plasm marker genes, and show that none of them localise asymmetrically within *Oncopeltus* oocytes or early embryos. Using multiple molecular and cytological criteria we provide evidence that PGCs form after cellularisation at the site of gastrulation. Functional studies of *vasa* and *tudor* reveal that these genes are not required for germ cell formation, but that *vasa* is required in adult males for spermatogenesis. Taken together, our results provide evidence that *Oncopeltus* germ cells may form in the absence of germ plasm, consistent with the hypothesis that germ plasm is a derived strategy of germ cell specification in insects.

Introduction

In sexually reproducing animals, only germ cells contribute genetic information to future generations. The germ line/soma separation is a cell fate decision shared across Metazoa (Buss, 1987). Despite the fundamental commonality of germ cell function in animals, the molecular mechanisms underlying germ cell specification are remarkably diverse across different taxa (Extavour and Akam, 2003; Extavour, 2007; Ewen-Campen et al., 2010; Juliano et al., 2010).

Primordial germ cells (PGCs) can be specified via different developmental mechanisms; here we call these “cytoplasmic inheritance” and “zygotic induction.” (We and others have previously referred to these mechanisms as “preformation” and “epigenesis” respectively (Nieuwkoop and Sutasurya, 1981; Extavour and Akam, 2003; Extavour, 2007). However, these terms can hold different meanings in other contexts of the history and philosophy of biology (see for example Callebaut, 2008). We therefore avoid them here in favour of more mechanistically descriptive terms). Cytoplasmic inheritance is characterised by the asymmetric formation of a specialised cytoplasmic region within the oocyte or early embryo, termed “germ plasm.” Germ plasm contains maternally provided mRNAs and proteins that are individually necessary and collectively sufficient for PGC formation. Cells that inherit germ plasm during embryogenesis acquire germ line fate. The best understood example of cytoplasmic inheritance occurs in *Drosophila melanogaster*, where germ plasm is maternally synthesised, localised to the posterior of the oocyte during oogenesis, and subsequently incorporated into PGCs (pole cells) during cellularisation. Removing pole cells after their formation, or compromising the molecular components of germ plasm, leads to loss of PGCs and sterility in adulthood (reviewed by Mahowald, 2001). In contrast, zygotic induction of PGCs takes place later in development and requires signalling from neighbouring somatic cells to induce germ line fate. This mode of PGC development is exemplified by *Mus musculus*, wherein PGCs develop from a subset of presumptive mesodermal cells after the segregation of embryonic and extraembryonic tissues in response to local signalling (reviewed by Magnusdottir et al., 2012).

Across Insecta, germ plasm has been almost exclusively reported in taxa nested within Holometabola (“higher” insects, which undergo complete metamorphosis)

including *D. melanogaster* (reviewed by Kumé and Dan, 1968; Anderson, 1973; Nieuwkoop and Sutasurya, 1981), and in only three species belonging to the sister assemblage to the Holometabola (see below). Thus, although the vast majority of our knowledge of insect germ cell development comes from studies of germ plasm in *D. melanogaster*, this mode of germ cell specification is likely a derived feature of Holometabolous insects and their close sister taxa.

Our present knowledge of PGC specification in basally branching insects (Hemimetabola) is based almost entirely on classical histological studies of insect development conducted over the past 150 years. Nearly all of these report that PGCs arise late in embryogenesis, raising the possibility that they may be specified through inductive mechanisms (Wheeler, 1893; Heymons, 1895; Hegner, 1914; Nelsen, 1934; Roonwal, 1937). Experimental approaches to discovering germ plasm in Hemimetabola are limited, but a study involving destruction of the germ rudiment via irradiation in the cricket *Gryllus domesticus* (Schwalm, 1965) showed that no specific region of early embryos in this species contains a germ line determinant. Functional genetic tests of genes that may specify germ cells in Hemimetabola have been performed in only one insect, the cricket *Gryllus bimaculatus*. In this cricket, the conserved germ line markers *vasa* and *piwi* are dispensable maternally and zygotically for PGC formation (Ewen-Campen et al., submitted). Most evidence available for the Hemimetabola therefore suggests the absence of germ plasm and the operation of zygotic PGC specification mechanisms.

Exceptions have been reported, however, in some members of the Paraneoptera, an assemblage of insect orders (including Hemiptera [true bugs], Psocoptera [book lice], and Thysanoptera [thrips]) that collectively form the sister group to Holometabola (Yeates et al., 2012). Cytological studies of three paraneopteran species, a book louse (Psocoptera, Goss, 1952), a thrip (Thysanoptera, Heming, 1979) and an aphid (Hemiptera, Chang et al., 2009) suggested the presence of germ plasm in oocytes or early embryos. Expression studies of *vasa*, *piwi* and *nanos* expression during asexual development of the pea aphid *Acyrtosiphon pisum* suggest the presence of a germ plasm (Chang et al., 2006; Chang et al., 2007; Chang et al., 2009; Lu et al., 2011). However, *A. pisum* embryogenesis is highly modified relative to that of other hemimetabolous insects and even relative to other members of the same order (Miura et al., 2003). Studies of

embryogenesis in most other hemipterans describe absence of germ plasm and PGC origin after cellularisation from the blastopore region at gastrulation stages (Metschnikoff, 1866; Witlaczil, 1884; Will, 1888; Seidel, 1924; Mellanby, 1935; Butt, 1949; Sander, 1956; Kelly and Huebner, 1989; Heming and Huebner, 1994). We therefore wished to examine the expression and function of germ line genes in a hemipteran displaying embryological characteristics more representative of the order.

Here we characterise germ cell formation and migration in the milkweed bug *Oncopeltus fasciatus* (Hemiptera). We examine the expression of 19 molecular markers including *vasa*, *nanos*, and *piwi*, and test the germ cell function of three of these using RNA interference. We show that in striking contrast to *Drosophila*, transcripts of none of these genes localise asymmetrically within *Oncopeltus* oocytes or early embryos. We identify PGCs using multiple criteria, and show that neither *vasa* nor *tudor* are required for PGC specification or oogenesis in this species, but that *vasa* is required for spermatogenesis in adult males. These data show that the PGC specification role of *vasa* has diverged between *Oncopeltus* and the Holometabola, and suggest that *Oncopeltus* PGCs may form in the absence of maternally supplied germ plasm.

Results

Putative germ cells are first detectable in the late blastoderm stage

In contrast to *D. melanogaster*, classical studies of *Oncopeltus fasciatus* embryogenesis have not revealed a germ plasm in oocytes or early embryos, and instead first identify cells with cytological characteristics of PGCs at the posterior of the embryo at the end of the cellular blastoderm stage (Butt, 1949). We used semi-thin plastic sectioning and fluorescence microscopy to confirm these observations, and traced the development of these putative PGCs throughout gastrulation and germ band elongation (Fig. S1). Our observations of putative PGC formation in *Oncopeltus* were consistent with historical studies (Butt, 1949), showing that these cells first arise at the blastoderm posterior immediately prior to gastrulation (Fig. S1). Unlike pole cells in *D. melanogaster*, presumptive PGCs in *Oncopeltus* arise on the basal side of the blastoderm surface, adjacent to the yolk (Fig. S1G-H). In order to obtain further evidence that these

cells were PGCs and test for the presence of a maternally supplied germ plasm, we examined the expression of conserved germ line markers.

Cloning Oncopeltus germ line markers

We cloned fragments of *vasa*, *nanos*, and *piwi* (Ewen-Campen et al., 2010) and confirmed that each was the best reciprocal BLAST hit to its respective orthologue in *D. melanogaster*. *vasa* was cloned using degenerate primers (Table S1). *nanos* and a single *piwi* gene were recovered from the *Oncopeltus* transcriptome, in addition to a single AGO-3 orthologue (an additional PIWI family protein belonging to a separate sub-family; not shown). We believe it is unlikely that *Oncopeltus* possesses additional orthologues of these genes because (1) the *Oncopeltus* ovarian and embryonic transcriptome, which is nearly saturated for gene discovery and has an average coverage of 23X (Ewen-Campen et al., 2011), contained only one orthologue of each gene; and (2) degenerate PCR for *vasa* using primers flanking the conserved DEAD box helicase domain (Rocak and Linder, 2004) recovered only a single *vasa* orthologue.

Phylogenetic reconstruction confirmed that *Oncopeltus vasa* is nested within other insect *vasa* genes (Fig. S2A), and that *Oncopeltus piwi* belongs to the PIWI sub-family containing the *Drosophila* genes *piwi* and *aubergine* (which are *Drosophila*-specific duplications) (Fig. S2B). The portion of animal Nanos proteins with conservation sufficient for confident alignment (48 amino acids) is too short to yield significant phylogenetic signal (Fig. S2C, note low support values), but *Oncopeltus* Nanos does contain the diagnostic 2x(CCHC) zinc finger domain found in all Nanos orthologs (Fig. S2D).

Our analysis of the *Oncopeltus nanos* sequence produced an unexpected result: we found that a stop codon is present 771 bp upstream of the first CCHC zinc finger domain, although no methionine is found anywhere in this region. This is unlikely to be a sequencing error, as it was identified with high coverage (22 reads/bp at this position) in the transcriptome (Ewen-Campen et al., 2011) and confirmed using Sanger sequencing of independent clones generated from a different cDNA pool than that used to generate the transcriptome. Furthermore, repeated attempts at 5' RACE using a third independent DNA pool failed to amplify a start codon. Several lines of evidence confirm that this

Oncopeltus nanos sequence represents a highly expressed mRNA and is therefore unlikely to be a pseudogene: it was recovered from a transcriptome made solely from poly(A)-RNA, and is detected via both RT-PCR (Ewen-Campen et al., 2011) and in situ hybridisation (see below). We hypothesise that a large, unspliced intron downstream of the start codon may have been present in our mRNA preparations. Alternatively, given that the length of the predicted translated region upstream of the first CCHC zinc finger domain (266 amino acids) is within the range of known arthropod Nanos orthologues (95 to 332 amino acids) (Wang and Lehmann, 1991; Curtis et al., 1995; Calvo et al., 2005; Lynch and Desplan, 2010), it may be that the *Oncopeltus* Nanos N terminus has a non-methionine start codon. Although rare, eukaryotic non-AUG translation initiation can occur in nuclear-encoded genes, including developmentally relevant genes (Hellen and Sarnow, 2001), and can be recognized by insect ribosomes (Sasaki and Nakashima, 2000; Jan et al., 2001). In the absence of a complete genome sequence we cannot distinguish between these hypotheses. Despite this uncertainty, we report *nanos* transcript expression here for the sake of completeness.

vasa, nanos, and piwi transcripts do not localise asymmetrically in ovaries

The distinct cytoplasm inherited by early-specified PGCs in multiple organisms, including *D. melanogaster*, *Caenorhabditis elegans*, *Xenopus laevis* and *Danio rerio*, contains transcripts of the highly conserved *piwi*, *vasa* and *nanos* gene families. Of these, only *nanos* mRNA is asymmetrically localised to *D. melanogaster* germ plasm, while *piwi* and *vasa* transcripts are ubiquitous throughout the fly oocyte and embryo. However, in several other organisms *vasa* orthologue transcripts are asymmetrically localised germ plasm components (reviewed by Ewen-Campen et al., 2010).

To test whether any of these transcripts were asymmetrically localised to putative germ plasm in *Oncopeltus* oocytes, we conducted in situ hybridisation on adult ovaries. The structure of *Oncopeltus* ovaries is typical of Hemiptera and several other insect orders but differs remarkably from that of *Drosophila* (Fig. 1A) (Büning, 1994). Rather than each oocyte developing together with its own complement of 15 nurse cells as in *Drosophila*, all oocytes in *Oncopeltus* ovarioles share a common pool of syncytial nurse cells located at the anterior of each ovariole in a region termed the “tropharium” (Fig.

1A1). The nurse cell syncytium connects to all oocytes via elongated, microtubule-rich tubes called “nutritive tubes” (Hyams and Stebbings, 1979; Harrison et al., 1991) through which maternal factors, including mRNA, proteins and mitochondria, are transported to developing oocytes (Fig. 1A2-A3) (Stebbing et al., 1985; Stebbings and Hunt, 1987; Anastasi et al., 1991; Hurst et al., 1999; Stephen et al., 1999).

vasa, *nanos*, and *piwi* were expressed at high levels in *Oncopeltus* nurse cells and oocytes of all stages, but at no stage of oogenesis did any of these three transcripts localise asymmetrically within oocytes (Fig. 1B-D). Expression was detected in nurse cells, resting oocytes, nutritive tubes, and developing oocytes, suggesting that these transcripts are synthesised in the nurse cells and subsequently transported to oocytes via nutritive tubes (Fig. 1B-D). *nanos* and *piwi* were expressed throughout the tropharium (Fig. 1C, D), in contrast to *vasa*, whose expression was primarily in nurse cells of the posterior tropharium, resting and developing oocytes (Fig. 1B). In late stage oocytes, expression remained ubiquitous (not shown), similar to the expression in just-laid eggs (see below).

In situ screen of conserved *Drosophila* germ plasm markers fails to reveal a germ plasm in *Oncopeltus*

The expression of *piwi*, *vasa* and *nanos* suggests that a maternally localised germ plasm containing transcripts of these genes is not present in *Oncopeltus* oocytes. However, a functional germ plasm that contains gene products other than those encoded by these three genes could be present in oocytes or early embryos. To explore this possibility, we examined the expression of 14 additional genes whose transcripts are enriched in the germ plasm and germ cells of *Drosophila* (Table S1) (Tomancak et al., 2002; Lécuyer et al., 2007; Tomancak et al., 2007), that were also recovered from the *Oncopeltus* ovarian and embryonic transcriptome (Ewen-Campen et al., 2011) based on best reciprocal BLAST hit analysis with the *Drosophila* proteome (Zeng and Extavour, 2012). Although several of these genes do not have documented mutant phenotypes for germ cell formation in *Drosophila* (Table S1), all are expressed at high levels in germ plasm and/or pole cells and are therefore molecular markers for germ plasm in

Drosophila. We reasoned that if *Oncopeltus* possessed germ plasm it would likely be revealed by the transcripts of at least one of these genes.

In addition, we examined the expression of *boule* and *tudor*, which have widely conserved functions in germ cells across Metazoa (Eberhart et al., 1996; Ewen-Campen et al., 2010; Shah et al., 2010). *tudor* is one of 23 Tudor domain-containing proteins in *Drosophila* (Ying and Chen, 2012), but there is no evidence that loss of function of other Tudor domain-containing genes have grandchildless phenotypes in *Drosophila* (Handler et al., 2011; Pek et al., 2012). We therefore focus only on the expression and function of the orthologue of *Drosophila tudor* (CG9450). We examined *boule* and *tudor* transcript expression throughout oogenesis and embryogenesis through mid-germ band stages.

None of these 16 transcripts localised asymmetrically in ovaries (Fig. S3). Instead, like *vasa*, *piwi* and *nanos* (Fig. 1), all of these genes were expressed ubiquitously throughout oogenesis. Half of the genes examined (*sra*, *CycB*, *Bsg25D*, *Uev1A*, *CG16817*, *Unr*, *mael* and *tud*) were expressed, like *vasa* (Fig. 1B), in nurse cells adjacent to resting oocytes, as well as in the resting and early oocytes themselves (Figs. 2A; S3B-H). Five genes (*Gap1*, *eIF5*, *bel*, *orb* and *boule*) were, like *piwi* and *nanos* (Fig. 1C-D), strongly expressed in all nurse cells of the tropharium (Figs. 2G; S3I-L). Two genes (*cta* and *Tao*) were expressed in resting and early oocytes but barely at all in the tropharium (Fig. S3M-N), suggesting that these genes may be transcribed by resting oocyte nuclei rather than by nurse cells. Finally, *aret* (aka *bruno*), which is a translational regulator of Oskar in *Drosophila* (Kim-Ha et al., 1995; Webster et al., 1997), was expressed in nurse cells of the posterior tropharium and in early stages of oogenesis but excluded from resting oocytes (Fig. S3O), suggesting that it is transcribed by oocyte nuclei after the onset of oogenesis. In summary, although transcripts of most of these genes are likely to be supplied maternally to oocytes, they are not asymmetrically localised within oocytes of any stage.

vasa, *boule* and *tudor* transcripts mark PGCs throughout embryogenesis but are not asymmetrically localised in early embryos

Although none of the genes examined showed asymmetric localisation during oogenesis of early embryogenesis, at late blastoderm stages many of the genes appeared

enriched at the posterior pit, where PGCs had been identified based on cytological criteria (Fig. S1) (Butt, 1949). However, because at this stage of development gastrulation begins at the posterior, this region of the blastoderm is multilayered. Upon close examination, we found that the apparent transcript enrichment was an artifact of tissue thickness for all genes except *vasa*, *tudor* and *boule*, whose transcripts appeared truly enriched in putative PGCs at late blastoderm/early gastrulation stages (Figs. 2D, F-H; 4N, S).

Strikingly, we found that *vasa*, *tudor* and *boule* marked PGCs from the time of their formation at cellular blastoderm stages, but that none of these genes' transcripts were asymmetrically localised prior to PGC formation. Immediately after egg laying, *vasa* transcripts were not localised asymmetrically but rather were ubiquitously distributed throughout the embryo (Fig. 2A). As energid nuclei reached the embryonic surface (Fig. 2B), cytoplasmic islands enriched with these transcripts were distributed evenly across the embryonic surface, remaining there as these energids divided to form the uniform blastoderm (Fig. 2C). Prior to posterior pit formation, *vasa* expression became restricted to putative PGCs at the embryonic posterior (Fig. 2D).

To visualise *vasa* expression in the developing PGCs in greater detail, we collected staged embryos in two-hour intervals over the period during which PGCs arise (19 to 27 hours after egg laying (AEL)), performed in situ hybridisation for *vasa*, and sectioned the embryos in plastic resin (Fig. 2E-H). During this eight-hour period, the blastoderm nuclei undergo two concurrent, dynamic processes: continuing cell divisions increase the nuclear density throughout the blastoderm, and the blastoderm nuclei move towards the posterior pole and ultimately into the yolk (Butt, 1949; Liu and Kaufman, 2004) (Fig. 2E'-H'). From 19-21 hours AEL, the ubiquitous *vasa* expression seen in early embryos remained unchanged (Fig. 2E-E'). However, from 21-23 hours AEL *vasa* expression became enriched in a subset of cells at the blastoderm posterior (Fig. 2F-F'). From 23-25 hours AEL, *vasa*-positive cells increased in density at the blastoderm posterior and began to move into the yolk (Fig. 2G-G'). This movement appeared passive, due to the formation of the posterior pit by invagination of the germ rudiment. However, in the absence of time-lapse data we cannot rule out the possibility of active PGC movement out of the blastoderm epithelium and towards the yolk. From 25-27 hours AEL, as the germ rudiment began its invagination into the yolk, *vasa*-positive cells

formed a distinct mesenchymal clump within the yolk at the posterior of the embryo (Fig. 2H-H’). During this and all following stages, in addition to the marked enrichment in PGCs, *vasa* transcripts are additionally observed ubiquitously at low levels throughout somatic tissue (Figs. 2-3).

Throughout all subsequent stages of germ band elongation and patterning, *vasa* continued to mark PGCs (Fig. 3). During early stages of germ band elongation prior to limb bud formation (~28-32 hours AEL) *vasa*-positive PGCs remained at the embryonic posterior on the dorsal surface of the newly forming mesoderm (Figs. 3A, B-B’). The PGC cluster then became pear-shaped from 32-42 hours AEL, as the anteriormost PGCs began to move towards the anterior of the embryos (Fig. 3A, C-C’). As the head lobes enlarged (36-40 h AEL), PGCs began to migrate anteriorly on the dorsal surface of the embryo and continued their migration during limb bud stages (40-44 h AEL) (Fig. 3A and C-C’). During appendage elongation stages (44-48 h AEL) PGCs split into distinct clusters spanning the midline in abdominal segments A4-A6, one cluster per segment. As appendage segmentation became morphologically distinct (48-52 h AEL), the segmental clusters split along the ventral midline into bilateral clusters in A4-A6.

tudor and *boule* were also expressed in PGCs at all stages in a pattern indistinguishable from that of *vasa* (Fig. 4), providing further evidence that the *vasa*-positive cells are *Oncopeltus* PGCs. None of the other genes we examined (Fig. S3), including *nanos* and *piwi* (Fig. 4), were enriched in PGCs at any stage.

Neither vasa nor tudor are required for PGC formation

Our gene expression analysis demonstrates that *vasa*, *tudor*, and *boule* are specifically expressed in PGCs beginning at the putative time of their specification at the embryonic posterior just prior to gastrulation. To determine whether these genes were required for PGC formation or development in *Oncopeltus*, we performed maternal RNAi (mRNAi) for each gene. We confirmed that mRNAi effectively reduced zygotic transcript levels in our experiments using RT-PCR (Fig. 5E). PGC presence or absence was determined with *in situ* hybridisation against PGC markers at ~40-54 hours AEL, when germ cells are visible on the dorsal mesoderm.

RNAi knockdown of *vasa* or *tudor* did not disrupt embryonic patterning or germ band development (Table S2), despite the widespread expression of these genes at early blastoderm stages (Figs. 2, 4), and their persistent low levels of expression in somatic cells even after PGC formation (Fig. 3, 4). Strikingly, germ cells were clearly present in both *vasa* (93.8%, n=16) and *tudor* (100%, n=20) knockdowns, suggesting that neither of these genes is required for PGC specification (Fig. 5A-C'). It is formally possible that residual *vasa* or *tudor* transcripts that may have escaped destruction by mRNAi could be sufficient to play an instructive role in PGC formation. However, we note that transcript levels of both genes in the progeny of injected mothers were barely detectable in the case of *vasa*, and undetectable in the case of *tudor*, when assessed with RT-PCR even as late as 4 days AEL (Fig. 5E). Moreover, even hypomorphic alleles of *tudor* (Schüpbach and Wieschaus, 1986) and *vasa* (Lasko and Ashburner, 1990; Schüpbach and Wieschaus, 1991; Liang et al., 1994) lead to loss of PGCs in *Drosophila*. We therefore hypothesise that in *Oncopeltus*, *vasa* and *tudor* are required neither maternally nor zygotically for germ cell specification, although they are expressed in the cells specified as PGCs.

To address the possibility of redundancy between these two genes, we performed double knockdown of *vasa* and *tudor*, which reduced transcripts of both genes to undetectable levels (Fig. 5E). Eggs laid by *vasa* + *tudor* double RNAi females had an increased rate of embryonic lethality relative to controls (Table 2; 47.4%, n=19 vs 10.3%, n=39), which may mean that these genes work together to play roles in somatic development. However, embryos that escaped this lethality still had PGCs (100%, n=10) (Fig. 5 D, D').

None of the knockdowns caused any qualitative or quantitative change in egg laying by injected females compared to controls, and ovaries of injected females showed neither morphological abnormalities nor signs of disrupted oogenesis (not shown). This indicates that, in contrast to *Drosophila* (Schüpbach and Wieschaus, 1991; Styhler et al., 1998; Tomancak et al., 1998; Johnstone and Lasko, 2004), *vasa* is not required individually or together with *tudor* for *Oncopeltus* oogenesis or egg laying.

boule is necessary for Oncopeltus oogenesis and embryonic survival

boule mRNAi caused a complete cessation of egg laying by injected females after four to five clutches (one clutch is laid every one to two days). In contrast, *vasa*, *tudor* and control mRNAi females continued to lay up to 12 clutches. Ovaries of *boule* dsRNA-injected females possessed only a few oocytes at early stages of oogenesis, and few or no mature oocytes (not shown), indicating a requirement for *boule* in the progression of oogenesis. Eggs laid by *boule* RNAi females displayed nearly complete embryonic lethality (81.8%, n=22) in all but the first clutch laid. (The first clutch of *Oncopeltus* eggs laid following mRNAi typically displays no abnormalities, as these eggs have developed their chorion by the time of injection and are therefore impervious to dsRNA (Liu and Kaufman, 2004).) This was a striking increase in embryonic lethality compared to *DsRed* controls (26.8%, n=190), *vasa* knockdowns (23.2%, n=198) and *tudor* knockdowns (5.6%, n=54). The oogenesis requirement for *boule* and resulting embryonic lethality thus prevented us from determining whether *boule* is required for germ cell specification in *Oncopeltus* and we do not further report on the role of *boule* on oogenesis in the present study.

vasa is required for Oncopeltus spermatogenesis

Given that in contrast to *Drosophila*, *vasa* is not required for germ cell specification or oogenesis in *Oncopeltus*, we wished to test for other possible functions of this gene. In mice, despite its expression in the embryonic PGCs of both sexes once they reach the genital ridge (Fujiwara et al., 1994; Diez-Roux et al., 2011), *vasa* is required not for PGC specification, but rather for gametogenesis in males (Tanaka et al., 2000). Similarly, we recently showed that *vasa* plays a role in spermatogenesis in the cricket *G. bimaculatus* (Ewen-Campen et al., submitted). We therefore asked whether *vasa* also functions during spermatogenesis in *Oncopeltus*.

The testes of *Oncopeltus* show an organisation typical of insect testes (Dumser, 1980), with stages of spermatogenesis located in an anterior-posterior progression. Unlike *Drosophila*, which has a single sperm tubule (testiole) per testis (Hardy et al., 1979), each *Oncopeltus* testis comprises seven testioles (Bonhag and Wick, 1953). In situ hybridisation for *vasa* showed that it is strongly expressed in secondary spermatogonia of each testiole, and at lower levels in early primary spermatocytes and post-spermatocyte

stages, but not in primary spermatogonia or somatic cells (Fig. 6A). Adult males injected with dsRNA against *vasa* displayed multiple abnormalities in spermatogenesis. Testioles of *vasa* RNAi males lacked clearly defined cysts and contained large numbers of small, dense nuclei in the anterior region (Fig. 6H-I, I'), which in controls contained only spermatocytes with large, pale nuclei (Fig. 6C, D, D'; S4E). The primary spermatogonial region of *vasa* RNAi testioles contained cysts of irregular size (Fig. 6I, arrowheads) with poorly defined cytoplasmic bridges (Fig. 6I', arrows). In the spermatocyte region *vasa* RNAi testioles contained large, poorly defined clusters of several hundred cells (Fig. 6J, arrowheads) at varying stages of spermatogenesis (Fig. 6J'). The nuclear morphology of cells in these cysts corresponded to spermatocyte (Fig. 6E; S4E) or early spermatid (Fig. 6F) stages, as well as shell stage-like nuclei (Fig. 6K-K') typical of the mid-stage spermatids of controls (Fig. 6F'). Cysts of wild type shell stage spermatids are no longer syncytial as the actin-rich cytoplasmic bridges disappear during spermatocyte stages (Fig. S4G). In contrast, the anterior shell stage-like nuclei in *vasa* RNAi testioles remained connected by cytoplasmic bridges (Fig. 6K', red arrows), consistent with precocious spermatid differentiation. Moreover, although they displayed clear shell stage nuclear morphology (Fig. 6K-K' red arrowheads), they were larger than wild type shell stage nuclei (Fig. 6F', red arrowheads), suggesting that they had begun spermatid differentiation as syncytial diploid cells without first proceeding through meiosis as in wild type. Finally, the posteriormost region of *vasa* RNAi testioles contained irregular groups of cells at mixed stages of late spermatid and spermatozoon differentiation (Fig. 6L), rather than the perfectly synchronised cysts of late spermiogenic stages seen in controls (Fig. 6G). These defects were observed in testes examined 28-29 days following injection of adult males, but are not artefacts of age, as testes of 10 week old wild type adult males showed normal progression through all stages of spermatogenesis (Fig. S4B).

Taken together, these data suggest that *vasa* is required for the maintenance of synchrony within cysts at multiple stages of spermatogenesis. In addition, *vasa* may be required for secondary spermatogonia to enter correct meiotic progression as spermatocytes, in the absence of which germ cells are nonetheless able to continue with subsequent stages of spermatogenesis.

Discussion

Oncopeltus germ cell formation

In several cases, analyses of molecular markers such as *vasa* mRNA have revealed the presence of a cryptic germ plasm that had eluded prior histological studies (Yoon et al., 1997; Tsunekawa et al., 2000; Wu et al., 2011). In *Oncopeltus*, we have shown that none of the transcripts of an extensive suite of conserved germ cell markers localise asymmetrically within oocytes or in early embryos (Figs. 1-2, 4; S3), including transcripts of genes that localise to and are required for the function of germ plasm in *Drosophila*. Gene products of at least one of these conserved germ line markers have been found in the germ plasm of every species where a germ plasm is known to exist (Ewen-Campen et al., 2010), although we note the important caveat that in *Drosophila*, several of these genes (*vasa*, *piwi*, and *tudor*) are localized as proteins rather than mRNAs. Thus, the lack of localisation of transcripts of any of these 19 genes during oogenesis or early embryogenesis suggests that *Oncopeltus* lacks germ plasm. Instead, our data support the hypothesis that *Oncopeltus* germ cells form in the absence of germ plasm, and are not present prior to the onset of posterior invagination at the end of the cellular blastoderm stage. We cannot, however, rule out the possibility that untested molecular markers, including protein products of the genes examined here, could be asymmetrically localised to a putative germ plasm in *Oncopeltus*.

While we provide multiple markers of PGCs, further experiments could be useful to confirm the identity of these cells. However, demonstration that these cells are functional PGCs via ablation experiments is complicated by the fact that they arise at the inner face of the blastoderm at the gastrulation center, so that their physical disruption would likely also compromise mesoderm formation and subsequent embryogenesis. Moreover, we note that while pole cell removal experiments in *Drosophila* result in sterility, pole cell removal in another insect with germ plasm, the wasp *Pimpla turionellae*, yields fertile adults despite the fact that these pole cells are bona fide PGCs in wild type embryos (Bronskill, 1959; Achtelig and Krause, 1971; Fleischmann, 1975). Further, we are currently unable to genetically ablate these cells and determine their effect on fertility, as our *vasa*, *tudor* and *vasa* + *tudor* RNAi double RNAi experiments

do not disrupt their formation (Fig. 5). Lineage tracing techniques that would permit tracking of the putative PGCs over the six-week period between PGC formation and sexual maturity are not currently available for *Oncopeltus*. These caveats notwithstanding, the molecular and morphological evidence that the cells we identify in this report are bona fide *Oncopeltus* PGCs is nevertheless comparable to that available for PGC identification in most studied animal species: (1) three conserved germ line genes, *vasa*, *tudor*, and *boule*, are specific germ cell markers in *Oncopeltus* (Fig. 1-4); (2) transcripts of these genes first become enriched in germ cells specifically at the time that these cells were previously reported to arise based on morphological and cytological criteria (Figs. 2-4) (Butt, 1949); and (3) cells with these molecular markers undergo migration and primordial gonad occupation (Figs. S1, 3-4) consistent with the well-documented behavior of PGCs in many other hemipterans (Seidel, 1924; Mellanby, 1935; Butt, 1949; Sander, 1956; Kelly and Huebner, 1989; Heming and Huebner, 1994).

Although the posterior location of germ cells at the time of their specification is superficially similar to that of pole cells in *Drosophila* and other Diptera, PGC specification and development in *Oncopeltus* differs in several important ways. First, while *Drosophila* pole cells form on the exterior of the posterior syncytial blastoderm before somatic cellularisation, *Oncopeltus* germ cells appear on the yolk side of the cellular blastoderm. Second, while *Drosophila* pole cells are the first cells in the embryo to cellularise (Huettnner, 1923), *Oncopeltus* germ cells arise after blastoderm cellularisation is complete (Butt, 1949). Third, because *Oncopeltus* is an intermediate-germ insect, only the gnathal and thoracic segments have been specified at the time that germ cells arise (Liu and Kaufman, 2004), whereas in the long-germ insect *Drosophila*, pole cells form posterior to the abdominal embryonic segments. Lastly, *Oncopeltus* germ cells form on the dorsal surface of the embryo, and remain on the yolk-facing surface of the mesoderm during their migration to the gonad primordium in anterior abdominal segments (Fig. 3). As a result, they do not undergo a transepithelial migration through the hindgut epithelium as in *Drosophila* (reviewed by Richardson and Lehmann, 2010).

The function of “germ line genes” in Oncopeltus

Our functional analysis led to the surprising discovery that neither *vasa* nor *tudor* play instructive roles in germ cell specification in *Oncopeltus*. Both of these genes are required for germ cell specification in *Drosophila* (Boswell and Mahowald, 1985; Schüpbach and Wieschaus, 1986) and other species (Sunanaga et al., 2007; Spike et al., 2008). However, *vasa* has widely divergent roles across Metazoa (reviewed by Yajima and Wessel, 2011), and in many cases is dispensable for PGC specification (Tanaka et al., 2000; Braat et al., 2001; Li et al., 2009; Özhan-Kizil et al., 2009). In several organisms it plays a role in adult gametogenesis (Tanaka et al., 2000; Ohashi et al., 2007; Salinas et al., 2007; Fabioux et al., 2009; Salinas et al., 2012; Ewen-Campen et al., submitted).

Intriguingly, we find that similar to the mouse and the cricket, *vasa* is required for spermatogenesis in adult *Oncopeltus* (Fig. 6), but not for oogenesis. This sex-specific function may relate to a putative role in stem cell function. As in other hemimetabolous insects (Büning, 1994), in *Oncopeltus* germ line stem cells are likely active in the apex of the testes (Schmidt and Dorn, 2004) but are not thought to be present in adult ovaries. One caveat to this hypothesis is that *vasa* transcript was not detected by in situ hybridisation in the primary spermatogonia (Fig. 6A), although it may be present at very low levels in those stem cells. Alternatively, given its strong expression in secondary spermatocytes and the defects in cyst integrity and synchrony caused by *vasa* RNAi (Fig. 6), *Oncopeltus vasa* may play a male-specific role in the onset or synchrony of meiosis. Consistent with a conserved role for *vasa* in bilaterian meiosis, male germ cells in *vasa* knockout mice arrest just prior to meiosis onset (Tanaka et al., 2000), and in human stem cell-derived germ cells *vasa* overexpression enhances meiotic progression (Medrano et al., 2012). *Oncopeltus vasa* RNAi leads to premature spermatid differentiation by some diploid secondary spermatocytes within a cyst, resulting in cyst asynchrony. In *Drosophila*, mutations are known that disrupt meiosis but do not prevent sperm formation (Davis, 1971), consistent with the hypothesis that spermiogenesis can be decoupled from meiotic status.

The evolutionary origins of germ plasm in insects

Together with recent molecular and classical histological data on germ cell specification in other insects, our results are consistent with the hypothesis that germ

plasm is a derived mode of germ cell specification which arose in the ancestor to holometabolous insects (Fig. 7) (Lynch et al., 2011; Ewen-Campen et al., 2012). The only other functional genetic analysis of germ line specification in a hemimetabolous insect to date (Ewen-Campen et al., submitted) has also provided evidence that maternally supplied posterior germ plasm is absent, and that *vasa* is dispensable maternally and zygotically for germ cell formation. Our data thus provide support that germ plasm-driven germ cell specification mechanisms operative in *Drosophila melanogaster* and *Nasonia vitripennis* are derived relative to the Hemimetabola (Fig. 7).

The ubiquitous distribution of germ cell markers in early *Oncopeltus* embryos and their subsequent enrichment to presumptive germ cells at the blastoderm posterior is reminiscent of *vasa* expression in the beetle *Tribolium* (Fig. 7) (Schröder, 2006; von Levetzow, 2008). Further taxonomic sampling, and functional studies in *Tribolium*, will be needed to determine whether the PGC specification mechanism in these two species may be the result of common ancestry (Fig. 7).

A large number of transcripts that localise to germ plasm in *Drosophila* are expressed ubiquitously in *Oncopeltus* oocytes and early embryos. This suggests that the evolution of germ plasm in Holometabolous insects involved a large-scale change in the localisation of many transcripts in the oocyte. We propose that this likely resulted from a change in the localisation of an upstream component capable of recruiting many downstream transcripts, rather than via the sequential evolution of distinct localisation mechanisms for individual transcripts. Studies on the genetic mechanism of evolutionary redeployments of multiple downstream genes have largely focused on transcription factors, as individual transcription factors are capable of regulating large numbers of target genes (Hoekstra and Coyne, 2007; Moczek, 2008; Stern and Orgogozo, 2008; Craig, 2009). Interestingly, in the case of germ plasm, transcription factors are unlikely to have been key players in the mechanisms of evolutionary change for a number of reasons. First, regulation of germ line determinants is largely post-transcriptional (Arkov and Ramos, 2010; Richter and Lasko, 2011; Sengupta and Boag, 2012; Nusch and Eckmann, 2013). Second, germ plasm transcript function relies on their subcellular localisation (mediated via signals in their 3'UTRs) rather than their presence or absence (Rangan et al., 2009). Finally, unlike the key transcription factors identified as largely

sufficient to induce specific somatic cell fates (e.g. Akam, 1998; Kozmik, 2005; Baena-Lopez and Garcia-Bellido, 2006), there is no single conserved gene that is sufficient to confer germ cell fate across metazoans. The evolution of germ plasm may therefore serve as an example of how a novelty (asymmetrically localized germ plasm in the oocyte) arose via changes in RNA localisation rather than transcriptional regulation.

Materials and Methods

Animal culture

Oncopeltus fasciatus were cultured at 28°C as previously described (Ewen-Campen et al., 2011). Timing of embryonic events reported here may differ from that reported in other studies using lower rearing temperatures (e.g. Liu and Kaufman, 2004).

Cloning and phylogenetic analysis

Total RNA was extracted from mixed-stage embryos and ovaries using TRIzol (Invitrogen) and used for first strand cDNA synthesis with qScript cDNA SuperMix (Quanta BioSciences). An *Oncopeltus vasa* fragment was cloned using degenerate primers (Table S3). *nanos* and *piwi* fragments were obtained from the *Oncopeltus* transcriptome (Ewen-Campen et al., 2011). Fragments were extended using RACE PCR (SMART RACE cDNA kit, Clontech), and used as templates for DIG-labeled in situ probes and dsRNA fragments following sequence verification (Table S3). Genes for the in situ hybridisation screen (Tables S1, S3) were obtained from the *Oncopeltus* transcriptome (Ewen-Campen et al., 2011; Zeng and Extavour, 2012) and amplified using primers containing linker sequence (5'CCCGGGGC-3') enabling direct addition of a T7 site to the 3' end in a subsequent PCR reaction. Extended sequences are available from ASGARD (<http://asgard.rc.fas.harvard.edu/>) (Zeng and Extavour, 2012). All coding sequences reported in this study have been submitted to GenBank [accession numbers KC261571-KC261587] except for *orb* and *Uev1A*, for which we obtained only 3' UTR sequence.

Maximum-likelihood phylogenetic analysis was performed for *vasa*, *piwi*, and *nanos* as previously described (Ewen-Campen et al., 2012).

Tissue fixation and gene expression analysis

Embryos were fixed and stained as previously described (Liu and Kaufman, 2004; Erezylmaz et al., 2009; Kainz et al., 2011). Adult gonads were dissected in 1X PBS and fixed in 4% formaldehyde in 1X PBS for at least one hour. Antibodies used were mouse anti-alpha tubulin DM1A (Sigma) 1:50 and goat anti-mouse Alexa Fluor 568 (Invitrogen) 1:500 - 1:1000, and counterstains FITC-phalloidin (Invitrogen) 0.5 - 1 μ l and Hoechst 33342 (Sigma) 0.1 - 0.5 μ g/ml.

Plastic sectioning

In situ hybridisation and/or Sytox Green (Invitrogen) staining were performed prior to embedding embryos in Durcupan ACM Fluka (Sigma), mixed at a ratio of 32 : 27 : 1 : 0.6 = components A:B:C:D. Embryos were dehydrated through 10-minute washes in each of 50%, 70%, 90%, 2 x 100% ethanol and 100% acetone, transferred to a 1:1 mixture of acetone : catalysed Durcupan, and left uncovered in a fume hood overnight. Embryos were individually transferred to fresh Durcupan in silicone molds (Electron Microscope Sciences # 70903) and oriented following a 30-minute initial hardening at 65°C. Resin blocks were baked for 24 hours at 65° C.

Block fronts were trimmed with a razor blade and sectioned at 5-6 μ M on a Leica RM2255 microtome with a high-profile knife holder using High-Profile disposable “diamond-edge” steel knives (C.L. Sturkey # D554D50). Sections were collected on water droplets on charged slides, dehydrated on a heat block, and mounted in Permount (Fisher Scientific).

Parental RNAi

dsRNA for all genes (Table S3) was prepared as previously described (Kainz et al., 2011). and resuspended in injection buffer (5mM KCl, 0.1 mM NaH₂PO₄) to a concentration of 2 μ g/ μ L. Male and female adults were injected three days after final molt with 5 μ L of 2 μ g/ μ L dsRNA using a Hamilton syringe and size 26 needles. Testes were collected from injected males 27-29 days after injection.

Reverse-transcription PCR

Half of each clutch laid by injected females was fixed for in situ hybridisation, and the other half was homogenised in TRIzol (Invitrogen) and stored at -80°C before isolation of total RNA. RNA was isolated separately from late blastoderm (24-29 hours AEL), early germ band (24-48 hours AEL) and late germ band (72-96 hours AEL) embryos laid by injected mothers. Genomic DNA was treated with Turbo DNase (Ambion) at 37°C for 30 minutes, followed by DNase heat-inactivation and phenol/chloroform extraction. cDNA was synthesised from 120 ng of each RNA sample using Superscript III Supermix (Invitrogen). PCR was performed using Advantage 2 DNA Polymerase from 1 µL of cDNA template and primers indicated in Table S1 at 60°C annealing temperature with 35 PCR cycles. RT-PCR results for samples of all three embryonic ages tested yielded indistinguishable results, indicating that maternal RNAi was effective at reducing zygotic transcripts in embryos at least up to four days AEL.

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Competing Interests

No competing interests declared.

Authors' Contributions

BE-C and CGE designed the research; BE-C, TEMJ and CGE performed experiments, collected and analysed data and wrote the manuscript; CGE obtained funding for the research.

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Figure Legends

Figure 1. Germ cell markers do not localise asymmetrically during oogenesis. (A)

Overview of a single *Oncopeltus* ovariole. *nc*: nurse cells; *o*: oocytes; *nt*: nutritive tubes; *f*: follicle cells. Boxed regions are enlarged in (A1-A3). (A1) Nurse cell syncytium containing polyploid nurse cell nuclei (white) connected by cytoplasmic bridges (green). (A2) Posterior tropharium containing oogonia (arrows) and resting oocytes (arrowheads). Caret indicates polyploid nurse cells in the anterior of this region. (A3) Nutritive tubes (*nt*) are actin-rich at the end that enters the anterior of each oocyte. Transcripts of *vasa* (B), *nanos* (C), *piwi* (D), *tudor* (E) and *boule* (F) are detected in nurse cells, nutritive tubes, and uniformly in oocytes. (G) A representative sense control (for *vasa*) is shown; sense controls for other genes were similar. Scale bar = 100 μ M in A, A3 and B (applies to C-D); 25 μ M in A1, A2. Anterior to the left in all panels.

Figure 2. *vasa* transcript expression first labels PGCs at late blastoderm stages. (A-D)

vasa transcript expression. (A'-D') Corresponding images of nuclear stains. (A, A') Immediately following fertilisation *vasa* is detected ubiquitously. Arrowhead: polar body. (B, B') In early cleavage stages *vasa* transcripts are associated with all energid nuclei. (C, C') During early syncytial blastoderm stages, *vasa* expression remains ubiquitous. (D, D') At cellular blastoderm stages (24-28 h AEL), *vasa* marks putative PGCs at the posterior pit (asterisk). (E-H) End-on perspective of the posterior of *Oncopeltus* embryos showing *vasa* expression during PGC formation. (E'-H') Corresponding images of nuclear stains. (E''-H'') Medial sections of *vasa*- (purple) and nuclear- (cyan) stained embryos at corresponding time points. (E-E'') In late syncytial blastoderm stages, *vasa* is expressed ubiquitously. (F-F'') In early cellular blastoderm embryos, *vasa* expression increases in some posterior cells (arrowheads in F, F'') while levels in the remainder of the blastoderm decrease (arrows in F, F''). (G-G'') At posterior germ band invagination *vasa*-positive cells (arrowheads) are the first cells to enter the yolk; *vasa* transcripts continue to be cleared from somatic tissue (arrows). (H-H'') As invagination proceeds *vasa* expression is largely restricted to PGCs (arrowhead) and nearly cleared from the soma (arrows). Scale bars = 100 μ M in A (applies to B-D, A'-D'); 500 μ M in E (applies

also to F-H’); and 50 μ M in E’’ (applies also to F’’-H’’). Anterior is to the left in A-A’’ and E’’-H’’.

Figure 3. *vasa* marks PGCs throughout migration. (A) *vasa* transcript expression during progressive stages of germ band development. Arrows indicate *vasa*-positive PGCs. (B) Medial section of an embryo at 28-32 h AEL, showing *vasa* in situ hybridisation (purple) and nuclear stain (cyan). Boxed region enlarged in (B’) shows PGCs in contact with ectoderm (*Ect.*) and the amnion (*Amn.*). (C) Medial section of an embryo at 36-40 h AEL, when PGCs (arrow) initiate migration along the mesoderm (*Mes.*). Boxed region enlarged in (C’). Scale bars = 200 μ M in A-C; 100 μ M in B’, C’. Anterior is up in A, left in B’, C’.

Figure 4. *Oncopeltus* PGCs express *tudor* and *boule*, but not *nanos* or *piwi*. In early embryos, expression of all four genes remains ubiquitous during energid proliferation (A, F, K, P) and blastoderm formation (B-C, G-H, L-M, Q-R). During posterior pit formation *nanos* is expressed throughout the length of the embryo (D), whereas *piwi* expression is reduced in the presumptive extraembryonic serosal tissue in the anterior of the embryo (I). Apparent posterior staining in (D) and (I) is the result of tissue thickness in that location, and is not specific to PGCs. *tudor* (N) and *boule* (S) transcripts become restricted to presumptive PGCs at the time of their specification. In germ band stage embryos, while *tudor* (O) and *boule* (T) mark presumptive PGC clusters, *nanos* is not detected (E), while *piwi* expression is ubiquitous (J). Scale bars = 500 μ M in A (applies also to B-D, G-I, K-N, P-S); 100 μ M in E, J; 200 μ M in O, T. Anterior to the left.

Figure 5. *vasa* and *tudor* are not required for PGC specification in *Oncopeltus*. (A-D) Bright field images of in situ hybridisations for PGC markers in different RNAi conditions; numbers indicate sample sizes and % of embryos with PGCs. Arrowheads indicate PGC clusters in abdominal segments A4-A6. (A’-D’) DIC images of the same embryos shown in (A-D) showing distinct PGC cluster morphology. (A, A’) In control embryos *vasa*-positive PGCs are visible on the dorsal surface of abdominal segments 4-5. PGCs are present in *vasa* RNAi (B, B’), *tudor* RNAi (C, C’), and double *vasa* + *tudor*

RNAi (**D**, **D'**) embryos. (**E**) RT-PCR validation of RNAi knockdown. Controls are animals injected with *DsRed* dsRNA. Expression of β -tubulin was analysed to confirm cDNA integrity and allow comparison of amounts of template per lane. Scale bar = 100 μ M. Anterior is up in all A-D'.

Figure 6. *vasa* is expressed in adult testes and required for spermatogenesis in *Oncopeltus*. Schematics indicate the region of the testis (A-C) or testiole (D-L) shown in each column. (**A**) *vasa* in situ of an adult wild-type testiole showing expression in the secondary spermatogonia. PSG: primary spermatogonia; SSG: secondary spermatogonia; PSC: primary spermatocytes; SSC: secondary spermatocytes; ST: spermatids undergoing spermiogenesis; SZ: spermatozoa. (**B**) *vasa* sense control probe. DIC optics (**C-F**, **F'**) and F-actin (green) and nuclear staining (white) (**D'**, **E'**) of control testioles reveals synchronized spermatogenic cysts separated by clear cyst boundaries (carets) (**C**), small cysts of spermatogonia (PSG) at the apex (**D-D'**), larger cysts of spermatocytes (SSC) posterior to the apex (**E-E'**; arrowhead in (**E'**) indicates somatic sheath cells associated with cysts of germ cells), early spermatids with round prominent nuclei (early ST) (**F**) and mid-stage spermatids with smaller, compact round nuclei (mid ST) (**F'**). (**G**) Late spermatid cysts in controls are synchronized in spermiogenesis; hollow arrowheads indicate somatic sheath cells. *vasa* RNAi testioles contain large masses of cells with heterogeneous nuclear morphologies (**H**; arrowheads). (**I**) PSG cysts are abnormal in shape and size, contain nuclei of multiple sizes (arrowheads), and (**I'**) have filamentous actin masses interspersed between nuclei (arrows) rather than clearly defined cytoplasmic bridges (compare with **D'**, arrows). (**J**, **J'**) Abnormal cysts contain clusters of small dense nuclei (arrowheads). (**K**, **K'**). Aberrant cysts retain cytoplasmic bridges at spermatid stages (red arrows), and contain nuclei with morphologies corresponding to different spermatogenic stages, including both early (white arrows; compare with **F**) and mid ST (red arrowheads; compare with **F'**) stages. (**L**) *vasa* RNAi late spermatid cysts are asynchronous, comprising multiple late spermatid and spermatozoon differentiation stages within a single cyst; cysts remain associated with sheath cells (hollow arrowheads). Scale bar = 200 μ M in A (applies to B); 100 μ M in C, H; 50 μ M in D-G, I-J, L; 25 μ M in F', K. Anterior is up in all panels.

Figure 7. Phylogenetic distribution of germ cell specification mechanisms and migration patterns across insects. Species shown are those for which data on the expression and/or function of molecular markers for germ cells during oogenesis and embryogenesis are available. Molecular data suggest absence of germ plasm in oocytes (circles) and early embryos (squares) of some Holometabola (*Tribolium*, *Apis*) and Hemimetabola (*Oncopeltus* and *Gryllus*), and somatic expression of *vasa* at post-blastoderm stages of development (diamonds) is not uncommon. In most species, PGCs undergo extensive migration from the site of specification to the gonad primordia (triangles). Data from this study and (Nakao, 1999; Mahowald, 2001; Donnell et al., 2004; Zhurov et al., 2004; Chang et al., 2006; Dearden, 2006; Juhn and James, 2006; Nakao et al., 2006; Schröder, 2006; Chang et al., 2007; Juhn et al., 2008; von Levetzow, 2008; Zhao et al., 2008; Chang et al., 2009; Khila and Abouheif, 2010; Lynch et al., 2011; Ewen-Campen et al., submitted). Phylogenetic relationships as in (Yeates et al., 2012).

Supplementary Material for “Evidence against a germ plasm in the milkweed bug *Oncopeltus fasciatus*, a hemimetabolous insect”

Ben Ewen-Campen, Tamsin E. M. Jones and Cassandra G. Extavour

The Supplementary Material accompanying this manuscript consists of the following files:

1. Supplementary Tables S1-S3
2. Supplementary Figure Legends
3. Supplementary Figures S1-S4

Supplementary Table 1. Genes included in *Oncopeltus* germ plasm *in situ* screen

<i>Drosophila</i> Gene Name	<i>Drosophila</i> <i>gene</i> symbol	<i>Drosophila</i> CG #	Transcript Expression in <i>Drosophila</i> Germ Plasm and PGCs			<i>Drosophila</i> germ line mutant phenotype (Molecular function)	Functional conservation outside <i>Drosophila</i>	References
			Germ Plasm	Pole Cells	Stage 9 PGCs			
<i>Boule</i>	<i>bol</i>	CG4760	No	No	No	Spermatogenesis defects (RNA binding)	Germ line expression/function across bilateria, often specific to spermatogenesis	[1, 2]
<i>tudor</i>	<i>tud</i>	CG9450	No*	No*	N.D.	Pole cell formation defects (tudor domain protein)	Germ line expression/function across bilateria	[3-5]

<i>orb</i>	<i>orb</i>	CG10868	Yes	Yes	No	Oogenesis defects (RNA binding)	N.D.	[1]
<i>sarah</i>	<i>sra</i>	CG6072	Yes	Yes	No	Oogenesis defects (Calcineurin regulation)	N.D.	[1]
Cyclin B	<i>CycB</i>	CG3510	Yes	Yes	N.D.	Fertility defects in both sexes (Cyclin protein)	N.D.	[1]
arrest (bruno)	<i>aret</i>	CG31762	Yes	Yes	Yes	Oogenesis defects (RNA binding)	N.D.	[1]
concertina	<i>cta</i>	CG40010	Yes	Yes	Yes	No germ line phenotype reported (G-protein alpha subunit)	N.D.	[1]
Gap1	<i>Gap1</i>	CG6721	Yes	Yes	Yes	No germ line phenotype reported (PH & C2-domain, Ras GTPase activation)	N.D.	[1]
eIF5	<i>eIF5</i>	CG9177	Yes	Yes	Yes	No germ line phenotype reported (translation initiation)	N.D.	[1]
<i>Blastoderm-specific gene 25D</i>	<i>Bsg25D</i>	CG14025	Yes	Yes	Yes	No germ line phenotype reported (Unknown)	N.D.	[1]
Uev1A	<i>Uev1A</i>	CG10640	Yes	Yes	Yes	No germ line phenotype reported (ubiquitin-protein ligase)	N.D.	[1]
CG16817	-	CG16817	Yes	Yes	Yes	No germ line phenotype reported (Unknown)	N.D.	[1]
Tao	<i>Tao</i>	CG14217	Yes	Yes	Yes	No germ line phenotype reported (Protein S/T kinase)	N.D.	[1]
<i>Upstream of N-ras</i>	<i>Unr</i>	CG7015	Yes	Yes	N.D.	No germ line phenotype reported (RNA and protein binding)	N.D.	[1]
Belle	<i>bel</i>	CG9748	N.D.	Yes	No	Oogenesis and spermatogenesis defects (ATP-dependent RNA helicase)	N.D.	[6]
maelstrom	<i>mael</i>	CG11254	No	Yes	Yes	Oogenesis and spermatogenesis defects (HMG-box DNA binding)	Germ line function in mouse	[1]

* Tudor protein is localised to both pole plasm and pole cells in *Drosophila*. N.D. = no data available.

Supplementary Table 2. Effects of RNAi on *Oncopeltus* PGC formation

RNAi	Total Scored	# embryos with non- specific defects* (%)	# surviving embryos with PGCs (%)
<i>DsRed</i>	39	4 (10.3%)	34 (97.1%)
<i>vasa</i>	16	0	15 (93.8%)
<i>tudor</i>	20	0	20 (100%)
<i>vasa and tudor</i>	19	9 (47.4%)	10 (100%)
<i>boule</i>	22	18 (81.8%)	4 (100%)

* “Non-specific defects” includes failure to develop a germ band as well as the formation of grossly defective germ bands, both of which ultimately resulted in embryonic lethality before 40-54 hours AEL, the time when we scored for PGCs. These embryos were not scored for PGC presence/absence.

Supplementary Table 3. Primers used for degenerate PCR, in situ hybridisation probe preparation, dsRNA preparation for RNAi, and RT-PCR. Sequence lengths are in nucleotides. F = forward; R = reverse.

Gene Name	degenerate primers		In situ probe length	Primers for in situ probe fragment (5' to 3')		dsRNA length	dsRNA primers (5' to 3')		RT-PCR amplicon length	RT-PCR primers (5' to 3')	
<i>vasa</i>	F	COGATCGCATGCTGGAYATGGGNTT	1403	F	AAAGGACTGGCAATGATGG	601	F	TGAGAGTATGACGAC	1392	F	GGAAGAGAAAGGGGACAAAGG
	R	GGTGGCGCGATGCKRTGNACRTA		R	AAACCTGGATCCCAAAATTC		R	TCCCGTCTGTTCAGAATCC		R	TCCCGTCTGTTCAGAATCC
<i>boule</i>			1287	F	ATTGAGGCACCAACTTCGAT	716	F	AGCCTCACCACCAGTATTCCG	614	F	ATTGAGATGAAACCTCCGGCCT
				R	AGGTGCCTAGGATTGGACT		R	AGGTGCCTAGGATTGGACT		R	AGTTCAGTGCCTCAGGGAAA
<i>tudor</i>			1215	F	GGTTAGCAAGCCTTGGAGTG	802	F	CCGAGAGTGTCTCAAGTTTCC	1876	F	TGCTCCAGTTGGTTCTTCC
				R	CACACCTGTTGCCATAATCG		R	AACTTGGTACGCCCTGTGGTC		R	CCACCAAAATCGCTTCTCATTT
<i>nanos</i>			843	F	GAAGGAACCCGTAGGGAA						
				R	ATAATCCCTGAAGTAGTGC						
<i>plwi</i>			1023	F	TGAAGAAATCAGAGCCAG						
				R	GATTGAGAGACGAGAAAGAA						
<i>arrest</i> (aka <i>bruno</i>)			703	F	ATGTTCACTGCCCTGGTAG						
				R	TACAGTGCCATACGGTTGA						
<i>belle</i>			725	F	GGTTGAGGAGCAAGACAAA						
				R	GCTCTGGTTGCGGAGTAAG						
<i>beta-tubulin</i>									199	F	TGATCCCTTACCTCGACACA
										R	CCCGCAAGGAAATCACTCT
<i>Blastoderm</i> -specific gene 25D (aka <i>Bsg25D</i>)			821	F	AGCTGGTGGAACCTCCAGAGA						
				R	TCAGTTCCTCCGAGTGCTTT						
<i>CG16817</i>			717	F	GCCATAGCTGGTTTCTCCAG						

Supplementary Figure Legends

Figure S1. Early embryogenesis of *Oncopeltus fasciatus* and morphological identification of putative PGCs. (A-C) The *Oncopeltus* syncytial blastoderm forms as a single layer of nuclei spread evenly across the surface of the yolk. (A) 8-12 hours after egg laying (h AEL) syncytially dividing nuclei are visible beneath the yolk surface. (B) Energid nuclei populate the yolk surface within 12 hours, and (C) undergo repeated mitosis and cellularisation to form a uniform cellular blastoderm by approximately 20 hours. Consistent with previous reports [7], we did not detect pole cell-like cells at any time during syncytial blastoderm or early cellular stages. (D) By 24-28 h AEL the posterior of the embryo has begun to invaginate into the yolk (arrows indicate direction of embryonic movements), forming the posterior pit (asterisk) where gastrulation takes place. This embryonic invagination is the beginning of the axial elongation process that will create the abdominal segments [8, reviewed by 9]. Immediately before posterior pit formation (~21 hours AEL), we observed putative PGCs on the inner surface of the blastoderm adjacent to the yolk (E-H'). (E) Medial section of a 19-21 h AEL embryo viewed with DIC optics and (E') stained with Sytox Green to reveal nuclei. Boxed region is enlarged in (F-H) and (F'-H'). (F, F') In 19-21 h AEL embryos, the early blastoderm is single-layered. (G, G') Between 21-23 h AEL, the embryonic posterior becomes multi-layered, and the first cells visible within the yolk mass are the presumptive PGCs (arrowheads). (H-H') By 27-29 h AEL the putative PGCs (arrowhead) have fully entered the yolk. These putative PGCs are visible as a mesenchymal cluster with large round, centrally located nuclei, directly adjacent to the epithelialized somatic cells of the posterior blastoderm (asterisk), which are columnar in shape with smaller, basally located nuclei. (I, I') As the germ band elongates and its posterior end invaginates into the yolk at 28-32 h AEL (arrows indicate direction of movement), the putative PGCs remain in a mesenchymal cluster at the germ band posterior. During early stages of germ band elongation (28-32 hours AEL), ongoing gastrulation produces mesodermal cells on the dorsal surface of the ectoderm (I-L'). The single-layered amnion (*Amn.*) is ventral to the ectoderm (*Ect.*); anterior mesoderm (*Mes.*) is on the dorsal surface of the ectoderm. Boxed region is enlarged in (J, J'). (J, J') Putative PGCs (arrowhead) form a cluster of cells at the posterior of the germ band, distinct from the adjacent ectoderm and amnion. (K, K') By 32-36 h AEL the embryo has nearly completed germ band elongation and its posterior end begins to curl towards the anterior of the egg within the yolk (arrows indicate direction of movement). Mesoderm now extends along its entire anterior-posterior extent. Boxed region is enlarged in (L, L'). (L, L') Putative PGCs (arrowhead) remain in a distinct cluster dorsal to the mesoderm and begins to migrate anteriorly along the dorsal surface of the mesoderm (see Fig. 3). Scale bars = 500 μ M in A-D, 100 μ M in E-L'. Egg anterior is to the left in all panels.

Figure S2. Phylogenetic analysis of *vasa*, *piwi*, and *nanos*. Best-scoring maximum likelihood cladograms are shown with bootstrap values from 2000 replicates at nodes. (A) *Oncopeltus* Vasa is a member of the Vasa family of RNA helicases, not the closely related PL10/belle proteins. (B) *Oncopeltus* Piwi is a member of the *piwi* clade of PIWI proteins, closely related to *Drosophila* Piwi and Aubergine. (C) Phylogenetic reconstruction fails to resolve the internal relationships of *nanos* genes, because the

conserved region of these proteins suitable for alignment (48 amino acids) is too short to provide sufficient phylogenetic signal. **(D)** A protein alignment of *Oncopeltus* Nanos protein with known orthologues demonstrates the presence of the diagnostic 2x(CCHC) zinc finger domain.

Figure S3. Expression of additional germ plasm candidate markers in *Oncopeltus* ovaries and embryos. Transcripts were chosen for analysis based on their expression in the germ plasm and PGCs of *Drosophila melanogaster* (Table S1). **(A)** Schematic figure showing the tissues depicted in subsequent panels. Embryonic ages shown in hours AEL. Coloured shading in ovariole schematic indicates spatial expression pattern of genes shown in boxes outlined in the corresponding colours. Blue = throughout entire tropharium in all nurse cells, as well as oogonia and resting oocytes; red = posterior nurse cells, oogonia and resting oocytes; green = oogonia and resting oocytes but absent from or very low in only posterior nurse cells of tropharium; magenta = posterior nurse cells of tropharium but not oogonia or resting oocytes. **(B-O)** Expression patterns of genes studied in ovaries (top of each panel), blastoderm stages from 0-28 hours AEL (arranged vertically along the left of each panel), and in mid-germ band stages (to right of each panel), when PGCs are easily discernable in embryos stained for *vasa*, *tudor* or *boule* (Figs. 3-4). None of the 14 genes shown here were asymmetrically localised within oocytes, or to PGCs in later stages of development. **(O)** *aret* was strongly expressed in a population of cells located at the posterior, dorsal surface of the head at germ band stages, perhaps implicating this gene in foregut development. Scale bars = 500 μ M for ovarioles and non-germ band embryos, 200 μ M for germ band embryos.

Figure S4. Spermatogenesis in wild type *Oncopeltus* adult testioles. **(A)** Sperm tubule (testiole) from a two-week old male. Germ line stem cells (primary spermatogonia) and their putative niche are located at the anterior apex of each testiole. Cysts of clonally related secondary spermatogonia, spermatocytes, spermatids and spermatozoa are arranged in order posterior to the niche. All cells of a given cyst proceed synchronously through all stages of spermatogenesis, and all cysts at the same position along the anterior-posterior axis of the testiole are also roughly synchronised with each other (A-B) [10]. PSG: primary spermatogonia; SSG: secondary spermatogonia; PSC: primary spermatocytes; SSC: secondary spermatocytes; ST: spermatids undergoing spermiogenesis; SZ: spermatozoa. **(B)** Testiole from a ten week-old male. All stages of spermatogenesis continue to progress normally, although a greater number of mature spermatodesms are present. **(C)** Primary spermatogonia divide mitotically to form cysts of two to eight cells, and remain connected by actin-rich cytoplasmic bridges (red arrows). **(D)** Secondary spermatogonia undergo six synchronous mitotic transit amplifying divisions to produce cysts of 64 nuclei that retain cytoplasmic bridges (green; red arrows) [10]. **(E)** Primary spermatocytes undergo the first meiotic division to produce 128 clonally related diploid cells, still connected by cytoplasmic bridges (red arrows). **(F)** Secondary spermatocytes undergo the second meiotic division synchronously; two cysts at anaphase (left) and metaphase (right) are shown. Each cyst is accompanied by a single large somatic sheath cell (arrowheads). **(G)** Nuclei of early “shell stage” spermatids appear hollow or shell-shaped [11] and begin to develop elongated tubulin-rich tails (red); cytoplasmic bridges are no longer present. **(H)** Mid-stage (“dot stage”) spermatid

nuclei condense (top cyst) and begin to elongate; tubulin-rich tails continue to elongate and actin-rich elongation complexes proceed posteriorly along the growing sperm tail (white arrows). **(I)** Late stage (“orzo” and “needle” stage) spermatid nuclei are further elongated. **(J)** Mature spermatozoa remain associated in spermatodesms containing all clonally related products of a single primary spermatogonium. Arrowheads in (F-J) indicate somatic sheath cells that are associated with each cyst. White = nuclei (Hoechst 33342), green = F-actin (FITC-phalloidin), red = anti-alpha Tubulin. Scale bars 100 μ M in A (applies also to B); 50 μ M in C-J. Anterior is to the top in all panels.

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